Pergamon

Tetrahedron Vol. 50, No. 23, pp. 6797-6804, 1994

0040-4020/94 $7.00+0.00

0040-4020(94)E0313-I

PSEUDOSTELLARINS A - C, NEW TYROSINASE INHIBITORY CYCLIC PEPTIDES FROM *PSEUDOSTELLARIA HETEROPHYLLA* [1)]

Hiroshi Morita, Takashi Kayashita, Hideyuki Kobata, Akira Gonda, Koichi Takeya and Hideji Itokawa*

Department of Pharmacognosy, Tokyo College of Pharmacy,
Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan

Abstract: New potent tyrosinase inhibitory cyclic peptides, pseudostellarins A - C, have been isolated from the roots of *Pseudostellaria heterophylla* and the structures were elucidated by extensive 2D NMR methods, chemical and enzymatic degradation and tandem MS spectroscopic analysis.

Recently a number of cyclic peptides with unique structures and biological activities have been isolated from natural origin. As part of our ongoing investigation of bioactive cyclic peptides from higher plants,[2)] we have isolated a several peptidic compounds from the roots of *Pseudostellaria heterophylla* (Caryophyllaceae), named pseudostellarins, showing potent tyrosinase and melanin production inhibitory activities. In this paper, we describe the isolation, structure elucidation and tyrosinase inhibitory activities of the peptidic compounds, named pseudostellarins A - C (**1** - **3**).

The methanolic extract of the roots of *P. heterophylla* was partitioned between *n*-BuOH and H_2O. The *n*-BuOH soluble material was subjected to Diaion HP-20 column (H_2O - MeOH) and 80% and 100% MeOH eluted fraction was chromatographed on a silica gel column, followed by HPLC on ODS to yield three peptidic compounds as colorless needles, named pseudostellarins A - C (**1** - **3**).

Pseudostellarin A (**1**), colorless needles, mp. 151 - 153 °C, $[\alpha]_D$ -118.7° (c 0.92, MeOH), showed a high-resolution FAB-MS spectral quasimolecular ion peak at *m/z* 502.2636 (M^++H, Δ +2.9 mmu), corresponding to molecular formula, $C_{25}H_{35}N_5O_6$. The IR absorptions at 3350 and 1660 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The pentapeptide nature of **1** was evident from its ^1H and ^{13}C NMR spectra, showing four amide NH and five amide carbonyl groups, as shown in Table 1. Further, the relatively high intensity of the molecular ion and

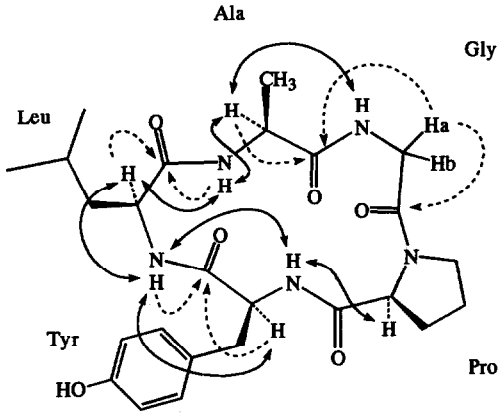

Fig. 1 Structure of Pseudostellarin A (1), Arrows show NOE relationship and dashed arrows show HMBC correlations.

the lack of terminal amino group protons in the ^{1}H NMR suggested **1** to be a cyclic pentapeptide. Amino acid analysis of the acid hydrolysate of **1** revealed the presence of one residue each on proline (Pro), alanine (Ala), glycine (Gly), leucine (Leu) and tyrosine (Tyr). The absolute stereochemistry of each amino acid in **1** was determined to be L-configuration by HPLC analysis, followed by derivatization of the acid hydrolysate with Marfey's reagent.[3]

Table 1. ^{1}H and ^{13}C NMR Signal Assignments of Pseudostellarin A (1) in pyridine-d$_5$.

		^{1}H NMR	^{13}C NMR				
assignment		δ_H (int. mult, J(Hz))	δ_C			δ_H	δ_C
Gly				Leu			
	α	3.91 (1H, dd, 6.2, 14.1;Ha)	42.34		α	4.48 (1H, br dd, 7.3, 16.3)	56.60
		4.34 (1H, dd, 4.8, 14.1;Hb)			β	1.90, 2.29 (each 1H, m)	40.03
	NH	9.26 (1H, br s)			γ	1.67 (1H, m)	25.19
	C=O		169.24		δ	0.82 (3H, d, 6.6)	21.87
Pro						0.88 (3H, d, 6.6)	22.97
	α	4.56 (1H, dd, 4.0, 7.5)	62.15		NH	9.41 (1H, d, 7.6)	
	β	1.94 (2H, m)	29.89		C=O		172.81
	γ	1.67 (2H, m)	24.82				
	δ	3.39, 4.16 (each 1H, m)	47.58				
	C=O		172.12				
Tyr				Ala			
	α	5.21 (1H, dd, 8.1, 16.2)	57.07		α	5.00 (1H, m)	49.83
	β	3.34 (1H, dd, 7.5, 13.6)	37.62		β	1.59 (3H, d, 7.1)	17.96
		3.45 (1H, dd, 8.5, 13.6)			NH	9.12 (1H, d, 8.4)	
	γ		128.57		C=O		174.56
	δ	7.39 (2H, d, 8.4)	130.95				
	ε	7.12 (2H, d, 8.4)	116.10				
	ζ		157.63				
	NH	8.12 (1H, d, 8.4)					
	C=O		172.43				

The NMR signals for individual amino acids, Pro, Ala, Gly, Leu and Tyr were readily assigned by extensive analysis of ^{1}H-^{1}H COSY and HMQC spectra.[4] The gross structure including the sequence of the amino acids for **1** was assembled by connecting the individual amino acids on the basis of connectivities observed in the HMBC experiment (Figure 1).[5] From the HMBC experimental results, the sequence was identified as Gly-Pro-Tyr-Leu-Ala. The deduced structure of

pseudostellarin A (**1**), cyclo[Gly-Pro-Tyr-Leu-Ala], was also in good agreement with the result of the NOE correlations in NOESYPH spectrum.[6]

Pseudostellarin B (**2**), colorless needles, mp. 167 - 169 °C, $[\alpha]_D$ -54.5° (c 0.32, MeOH), showed a molecular formula, $C_{33}H_{46}N_8O_8$, which was permitted by HR FAB MS spectrum, indicating 15 degrees of unsaturation. Amino acid analysis of **2** showed the presence of Pro × 2, Phe, Gly × 4 and Ile, which were confirmed to be all L-configuration by Marfey's derivatization, followed by HPLC analysis.[3]

The analysis of peptide sequences by tandem mass spectrometry (MS/MS) may now be considered a mature technique, routinely applied for applicative biochemical and pharmacological purposes.[7] Complete amino acid sequence information for **2** is obtainable by electrospray ionization (ESI)-MS/MS after selective enzymatic digestion.[8] No fragmentation of covalent bonds is typically evident in ESI mass spectra since the conditions required to obtain nearly complete desolvation are less severe than necessary for dissociation. Fragmentation can be induced in a separate step using collisionally activated dissociation (CAD), generally with MS/MS, by applying an additional stage of mass (m/z) selection prior to the dissociation step. Specific cleavage of amide bond at the aromatic amino acid units with α-chymotrypsin gave compound **4**, showing a molecular formula, $C_{33}H_{48}N_8O_9$, which was produced by cleavage of one amide bond at Phe. The sequencing of **4** was analyzed by ESI-MS/MS of m/z 701 $(M+H)^+$, the results showing in Fig. 2. From the MS/MS fragmentation, the peptide sequence could be deduced to be Gly-Ile-Gly-Gly-Gly-Pro-Pro-Phe. This sequence was also confirmed by extensive 2D NMR methods as described below. In the NMR spectra, the assignments of individual ^1H and ^{13}C signals of **2**, shown in Table 2, were made by the combination of ^1H-^1H COSY and HMQC spectra. Then, the above sequence was supported by NOE correlations in NOESYPH spectrum shown in Fig. 3 and the whole structure was established to be cyclo[Gly-Ile-Gly-Gly-Gly-Pro-Pro-Phe], named pseudostellarin B.

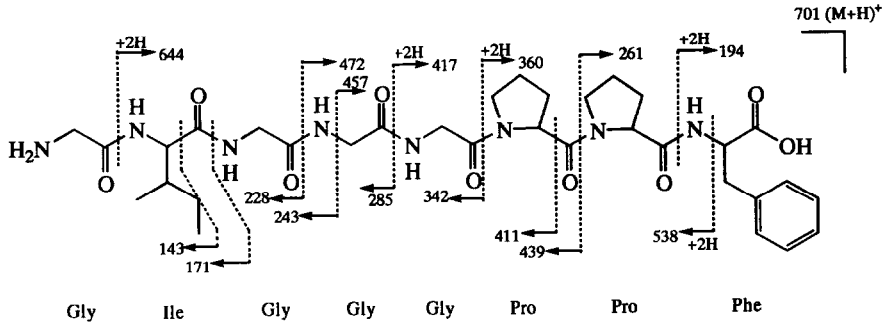

Fig. 2 ESI MS/MS fragmentation of m/z 701 $(M+H)^+$ ion of compound **4**

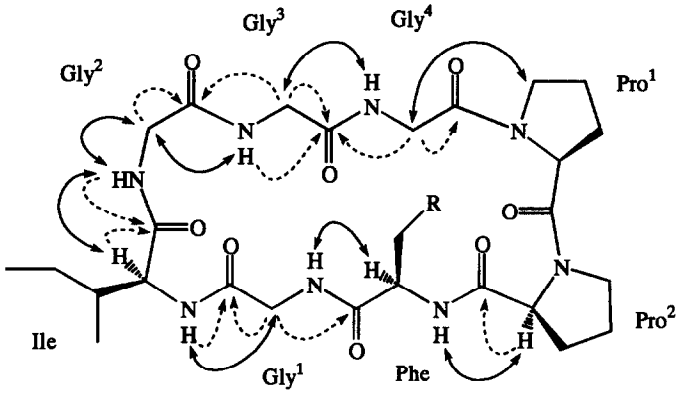

Fig. 3 Structure of Pseudostellarin B (2), R=phenyl, Arrows show NOE relationship and dashed arrows show HMBC correlations.

Table 2. ^{1}H and ^{13}C NMR Signal Assignments of Pseudostellarin B (2) in pyridine-d_5.

		^{1}H NMR	^{13}C NMR				
assignment		δ_H (int. mult, J(Hz))	δ_C			δ_H	δ_C
Gly[1]				Pro[1]			
	α	3.76 (1H, dd, 5.0, 16.6)	44.27		α	4.72 (1H, d, 7.3)	59.14
		4.64 (1H, dd, 7.2, 16.6)			β	1.70, 1.94 (each 1H, m)	28.46
	NH	8.68 (1H, br d, 5.9)			γ	1.67, 1.94 (each 1H, m)	24.39
	C=O		173.05		δ	3.36, 3.49 (each 1H, m)	46.78
Ile					C=O		170.84
	α	4.29 (1H, dd, 6.0, 9.5)	60.46				
	β	1.81 (1H, m)	34.79	Pro[2]			
	γ	1.19 (1H, m)	25.91		α	4.58 (1H, d, 8.3)	61.21
		1.67 (1H, m)			β	1.84, 1.94 (each, 1H, m)	32.02
	Me(γ)	0.74 (3H, t, 7.2)	15.45		γ	1.13, 1.40 (each, 1H, m)	21.88
	Me(δ)	0.92 (3H, d, 6.7)	10.47		δ	3.18, 3.36 (each, 1H, m)	47.19
	NH	8.09 (1H, d, 5.6)			C=O		172.89
	C=O		174.84				
Gly[2]				Phe			
	α	4.10 (1H, dd, 5.8, 16.8)	44.44		α	5.49 (1H, br t, 7.0)	55.50
		4.50 (1H, dd, 6.1, 16.8)			β	3.49 (1H, br d, 12.4)	38.12
	NH	10.46 (1H, t, 5.7)				4.02 (1H, dd, 3.8, 14.3)	
	C=O		170.02		γ		138.75
Gly[3]					δ	7.46 (2H, d, 7.2)	128.82
	α	4.01 (1H, dd, 5.2, 17.1)	43.45		ε	7.31 (2H, t, 7.2)	129.36
		4.68 (1H, dd, 7.5, 17.1)			ζ	7.24 (1H, t, 7.2)	126.95
	NH	8.68 (1H, br d, 5.9)			NH	8.98 (1H, br d, 7.2)	
	C=O		170.22		C=O		172.52
Gly[4]							
	α	3.66 (1H, dd, 2.9, 17.1)	41.70				
		4.76 (1H, dd, 7.8, 17.1)					
	NH	7.88 (1H, br d, 4.1)					
	C=O		168.53				

Pseudostellarin C (3), colorless needles, mp. 185 - 187 °C, $[\alpha]_D$ -39.1° (c 0.52, MeOH), showed a molecular formula, $C_{40}H_{61}N_8O_{10}$, which was permitted by HR FAB MS spectrum, indicating 15 degrees of unsaturation. Amino acid analysis of 3 showed the presence of Pro × 2, Phe, Ser, Thr, Gly and Leu × 2, which were confirmed to be all L-configuration by Marfey's derivatization, followed by HPLC analysis.

On the basis of the ^1H-^1H COSY and HMQC analysis of 3, all the ^1H and ^{13}C chemical shifts in DMSO-d_6 could be unambiguously determined to have Pro × 2, Phe, Ser, Thr, Gly and Leu × 2, being identical with the result of amino acid analysis. The HMBC spectrum providing proton-carbon long range couplings showed that the composition and position of the constituent amino acids were deduced to be as shown in Fig. 4. The sequence of the three structural units **A**, **B** and **C**, however, can not be combined. The sequence of this three units was established by HMBC and NOE correlations in pyridine-d_5; the HMBC correlation from the amide proton of Phe and α proton of Pro^2 to the carbonyl signal at δ 171.46 and the NOE correlation between the amide proton of Ser and α proton of Pro^1. Therefore, the whole structure of 3 was established to be cyclo[Gly-Thr-Leu-Pro-Ser-Pro-Phe-Leu], named pseudostellarin C. The complete assignments of the ^1H and ^{13}C signals in pyridine-d_5 solution were shown in Table 3.

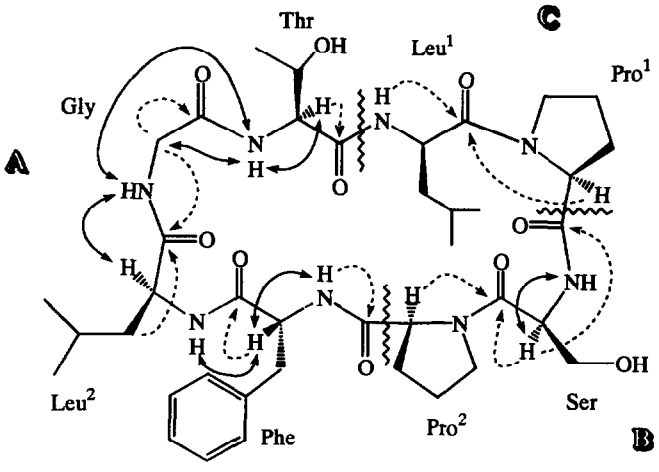

Fig. 4 Structure of Pseudostellarin C (3), Arrows show NOE relationship and dashed arrows show HMBC correlations in DMSO-d_6.

Table 3. ^{1}H and ^{13}C NMR Signal Assignments of Pseudostellarin C (**3**) in pyridine-d_5.

assignment		^{1}H NMR δ_H (int. mult. J(Hz))	^{13}C NMR δ_C	assignment		δ_H	δ_C
Gly				Ser			
	α	4.03 (1H, dd, 4.4, 16.9)	43.73		α	5.41 (1H, br dd, 9.7, 15.0)	52.44
		4.91 (1H, dd, 7.5, 16.9)			β	4.34 (1H, dd, 6.0, 9.9)	63.22
	NH	10.15 (1H, br s)				4.53 (1H, t, 9.9)	
	C=O		169.64		NH	8.74 (1H, d, *)	
Thr					C=O		172.73
	α	5.54 (1H, dd, 3.5, 9.8)	57.14	Pro[2]			
	β	4.62 (1H, dd, 3.5, 6.3)	68.73		α	4.54 (1H, d, 9.9)	61.61
	γ	1.55 (3H, d, 6.3)	19.71		β	1.98 (2H, m)	29.97
	NH	8.45 (1H, d, 9.8)			γ	1.67 (2H, m)	25.53[c]
	C=O		170.33		δ	4.05 (2H, m)	48.17
Leu[1]					C=O		171.46
	α	4.34 (1H, m)	54.57	Phe			
	β	1.67 (2H, m)	41.16[a]		α	5.67 (1H, br dd, 8.8, 14.2)	54.12
	γ	1.67 (2H, m)	25.45[b]		β	3.40 (1H, dd, 9.1, 13.6)	40.46
	δ	0.71 (3H, d, 5.9)	21.09			3.47 (1H, dd, 5.2, 13.6)	
		0.76 (3H, d, 5.9)	23.05		γ		138.23
	NH	10.15 (1H, br s)			δ	7.39 (2H, d, 7.2)	128.40
	C=O		172.73		ε	7.25 (2H, t, 7.2)	130.35
Pro[1]					ζ	7.20 (1H, t, 7.2)	126.56
	α	4.90 (1H, t, 7.5)	60.87		NH	7.92 (1H, d, 8.2)	
	β	2.20 (2H, m)	29.11		C=O		173.63
	γ	1.67 (2H, m)	24.99[c]	Leu[2]			
	δ	3.60 (2H, m)	47.85		α	4.70 (1H, m)	53.27
	C=O		172.73		β	1.67 (2H, m)	40.46[a]
					γ	1.67 (2H, m)	24.89[b]
					δ	0.64 (3H, d, 5.2)	22.07
						0.70 (3H, d, 5.2)	22.56
					NH	7.93 (1H, d, 6.2)	
					C=O		173.90

a-c) Assignments may be interchanged. * The signal was overlapped with the solvent peak.

Tyrosinase, which is distributed in animals and plants, can convert from tyrosine into melanin via a series of intermediate quinone and indolequinone compounds.[9] The melanin product formed is a complex heterogeneous biopolymer with many unusual properties; it is responsible for most of the pigmentation visible in the skin, hair and eyes of mammals. Tyrosinase in mammals is found exclusively within specialized dendritic cells called melanocytes. The initial reaction in the pathway of melanin formation from tyrosine through the catalytic action of tyrosinase involves the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Tyrosinase inhibitory activities (IC_{50}), showing the ratio of the production of DOPA, are 131 μM for **1**, 187 μM for **2** and 63 μM for **3**. This inhibitory concentration was more potent than that of arbutin (1.2 mM), well known as potent inhibitor of tyrosinase, and also cyclo(Pro-Tyr-Pro-Val) (1.5 mM), which was recently isolated from the lactic bacterium *Lactobacillus helveticus*.[10] Furthermore, pseudostellarin C showed potent inhibitory effect on the melanogenesis using cultured B16 melanoma cells (IC_{50} 171 μM).

Studies on the structure analyses and biological evaluations of a series of pseudostellarins and on the precise backbone conformation, their homogeneity and biological activity relationship are in progress.

Experimental

M.p.s were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1}deg cm^2 g^{-1}. FAB and high resolution mass spectra were taken with a VG Autospec spectrometer and ESI MS/MS spectrum with TSQ-700 spectrometer. IR spectrum was recorded on a JASCO A-302 spectrophotometer. High-pressure liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20mm i.d.×250mm and 30mm i.d.×250mm, GL Science Inc.) packed with 10μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying Dragendorff reagent. Proton and carbon spectra were recorded on Bruker spectrometers (AM400 and AM500) and processed on a Bruker data station with an Aspect 3000 computer. The 15 mg each sample of pseudostellarins A, B and C in a 5mm tube (0.5ml pyridine-d_5 or DMSO-d_6, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303K. NOESYPH experiments were acquired with mixing times of 0.6s. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 msec and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec.

Extraction and Isolation

The roots of *Pseudostellaria heterophylla* (10.0 Kg) were extracted with hot methanol at three times and concentrated to give crude extract. This extract was suspended with water and extracted with *n*-butanol, then concentrated to give *n*-butanol extract (167 g). This extract was suspended with water and subjected to Diaion HP-20 column (H_2O - MeOH). The 80% and 100% MeOH eluted fractions were chromatographed on a silica gel column with CH_2Cl_2 - MeOH solvent system. Finally 10% MeOH eluted fraction was purified by HPLC on ODS with 30% CH_3CN / 0.05% TFA solvent system to give pseudostellarins A (250 mg), B (600 mg) and C (450 mg).

Pseudostellarin A (**1**): Colorless needles, mp 151-153°C (from MeOH). FAB MS m/z: 502 (M^++H, Calcd for $C_{25}H_{35}N_5O_6$ 502.2665, Found 502.2636). $[\alpha]_D$ -118.7° (c 0.92, MeOH). ν_{max} (KBr) / cm^{-1} 3350 (NH) and 1660 (amide C=O). λ_{max} (MeOH) / nm 277 (ε 1460).

Pseudostellarin B (**2**): Colorless needles, mp 167-169°C (from MeOH). MS m/z: 683 (M^++H, Calcd for $C_{33}H_{47}N_8O_8$ 683.3518, Found 683.3494). $[\alpha]_D$ -54.5° (c 0.32, MeOH). ν_{max} (KBr) / cm^{-1} 3350 (NH) and 1650 (amide C=O). λ_{max} (MeOH) / nm 257 (ε 420).

Pseudostellarin C (**3**): Colorless needles, mp 185-187°C (from MeOH). MS m/z: 813 (M^++H, Calcd for $C_{40}H_{61}N_8O_{10}$ 813.4510, Found 813.4546). $[\alpha]_D$ -39.1° (c 0.52, MeOH). ν_{max} (KBr) / cm^{-1} 3350 (NH) and 1640 (amide C=O). λ_{max} (MeOH) / nm 285 (ε 670).

Acid Hydrolysis of **1** - **3**

Solutions of **1** - **3** (each containing 1 mg of peptide) in 6N HCl were heated at 110°C for 24h. After cooling, each solution was concentrated to dryness. The hydrolysates were soluble in 0.02N HCl and applied to the analysis by an amino acid analyzer.

Absolute Configuration of Amino Acids[3)]

Solutions of **1** - **3** (each containing 1 mg of peptides) in 6N HCl were heated at 110° for 12h. After being cooled, each solution was concentrated to dryness. The residue was soluble in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1M $NaHCO_3$ at 35° for 1h. After being cooled, 2M HCl was added and then concentrated to dryness. This residue was subjected to HPLC (Lichrospher 100, RP-18 (10μm), Merck), flow rate 1 ml/min, detection 340nm,

solvent : 10 - 50% CH_3CN / 50mM triethylamine phosphate (TEAP) buffer. The t_R values were L-Ser 20.25, L-Thr 21.75, L-Ala 26.08, L-Pro 28.04, L-Tyr 31.63, L-Ile 40.46, L-Phe 40.79 and L-Leu 41.08 min, respectively.

Enzymatic Hydrolysis of **2**

α-chymotrypsin (500 μg dissolved in 50 μl of 0.001% HCl, Wako Pure Chemical Industries, substrate-enzyme ratio, 400:1) was added to **2** (10 mg) in NH_4HCO_3 solution (1%, 0.9 ml) and the digestion was performed at 35 ℃ with the pH maintained at 8.0 by the manual addition of 0.1N HCl. After 24 h the reaction was stopped by adjusting the solution to pH 2.2 with 1N HCl. The digestion mixture was lyophilized to dryness and hydrolysates were subjected to HPLC (Inertsil PREP-ODS column, 20mm i.d.×250mm, GL Science Inc., packed with 10μm ODS, eluted with 30% CH_3CN / 0.05%TFA) to give compound **4** (8 mg), amorphous powder, $[\alpha]_D$ -43.0° (c 0.23, MeOH).

Enzyme Assay of Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity was assayed by the dopachrome method[9] with slight modification. Twenty five μl of mushroom tyrosinase solution (1000 U/ml), 37.5 μl of L-tyrosine (2.5 mM), 37.5 μl of 0.4 M HEPES buffer (pH 6.8) and 50 μl of ethanol with or without the test specimen, were mixed in a 96-well plate, incubated at 37°C for 15 minutes, and the absorbance of each well measured at 475 nm with a BIO-RAD plate reader (Model 3550), before and after incubation. The percentage inhibition of tyrosinase was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = \{(D-C)-(B-A)\}/(D-C) \times 100$$

A and B represent the absorbance in the presence of the test specimen before and after incubation, respectively. C and D represent the absorbance without the test specimen, before and after incubation, respectively.

Acknowledgments: We are grateful to Miss Sachiko Shirota, Dr. Ritsuo Aiyama and Mr. Minoru Ichioka of Yakult Central Institute for Microbiological Research for the biological evaluations.

References and Notes

1) Cyclic Peptides from Higher plants. VI.; Part V. H. Morita, H. Kobata, K. Takeya and H. Itokawa, Tetrahedron Lett., in press.
2) H. Itokawa and K. Takeya, Heterocycles, 1993, **35**, 1467; H. Morita, S. Nagashima, K. Takeya and H. Itokawa, Chem. Pharm. Bull., 1993, **41**, 992; H. Morita, S. Nagashima, O. Shirota, K. Takeya and H. Itokawa, Chem. Lett., 1993, 1877; H. Morita, S. Nagashima, K. Takeya, H. Itokawa and Y. Iitaka, J. Chem. Soc., submitted.
3) P. Marfey, Carlsberg Res. Commun., 1984, **49**, 591.
4) A. Bax and S. Subramanian, J. Magn. Reson., 1986, **67**, 565.
5) A. Bax and M. F. Summers, J. Am. Chem. Soc., 1986, **108**, 2094.
6) G. Bodenhauser, H. Koger and R. R. Ernst, J. Magn. Reson., 1984, **58**, 370.
7) "Tandem Mass Spectrometry," ed. by F. W. McLafferty, Wiley, New York (1983).
8) A. P. Bruins, T. R. Corey and J. D. Henion, Anal. Chem., 1987, **59**, 2642.
9) V. J. Hearing, Methods in Enzymology, 1987, **142**, 154.
10) H. Kawagishi, A. Somoto, J. Kuranari, A. Kimura and S. Chiba, Tetrahedron Lett., **34**, 3439 (1993).

(*Received in Japan* 18 *February* 1994; *accepted* 28 *March* 1994)